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Mutational Analysis of the APC/β-Catenin/Tcf Pathway in Colorectal Cancer[1]

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Abstract

Mutation of the adenomatous polyposis coli (*APC*) tumor suppressor gene initiates the majority of colorectal (CR) cancers. One consequence of this inactivation is constitutive activation of β-catenin/Tcf-mediated transcription. To further explore the role of the APC/β-catenin/Tcf pathway in CR tumorigenesis, we searched for mutations in genes implicated in this pathway in CR tumors lacking *APC* mutations. No mutations of the γ-catenin (*CTNNG1*), GSK-3α (*GSK3A*), or GSK-3β (*GSK3B*) genes were detected. In contrast, mutations in the NH_2-terminal regulatory domain of β-catenin (*CTNNB1*) were found in 13 of 27 (48%) CR tumors lacking *APC* mutations. Mutations in the β-catenin regulatory domain and APC were observed to be mutually exclusive, consistent with their equivalent effects on β-catenin stability and Tcf transactivation. In addition, we found that *CTNNB1* mutations can occur in the early, adenomatous stage of CR neoplasia, as has been observed previously with *APC* mutations. These results suggest that *CTNNB1* mutations can uniquely substitute for *APC* mutations in CR tumors and that β-catenin signaling plays a critical role in CR tumorigenesis.

Introduction

Inherited mutations of the *APC*[4] tumor suppressor gene cause familial adenomatous polyposis and acquired *APC* mutations initiate the majority of human CR cancers (reviewed in Ref. 1). More than a half dozen proteins, including β-catenin (2, 3), γ-catenin (4, 5), GSK-3β (6), EB1 (7), and hDLG (8), have been shown to associate with APC, but their importance to the neoplastic process remains unclear. Recently, however, the identification of increased β-catenin/Tcf signaling in CR cancers and the ability of wild-type APC to inhibit this activity focused attention on β-catenin as a critical mediator of APC function (9, 10). The inhibitory activity of APC likely results from its ability to form a macromolecular complex with β-catenin and GSK-3β (6), thereby facilitating degradation of β-catenin (11, 12). Accordingly, in a subset of cancers possessing intact APC, activation of β-catenin/Tcf signaling appears to be due to dominant activating β-catenin mutations that render the protein insensitive to APC/GSK-3β-mediated degradation (10, 13). Three APC-associated proteins, β-catenin, γ-catenin, and GSK-3β, have been linked to the APC/β-catenin/Tcf pathway by biochemical and genetic studies in diverse organism (reviewed in Ref. 14). To more fully define the role of the APC/β-catenin/Tcf pathway in human tumorigenesis, we performed mutational analyses of several components of this pathway in CR tumors.

Materials and Methods

Patient Samples. Our initial study included a panel of 40 primary human CR tumors in which the entire coding region of *APC* had been previously sequenced (15). These microdissected tumors included 24 adenocarcinomas (material was not available from one of the original samples, C3) and 16 adenomas. Five of these carcinomas had previously been analyzed for β-catenin mutations (10). An additional panel of 33 adenocarcinomas were passaged *in vitro* or as xenografts in nude mice to further minimize interference by contaminating nonmalignant cells. These carcinomas were selected solely on the presence of MIN and the availability of sample. β-catenin mutations had previously been found in two of these carcinomas (10).

Mutational Analysis of β-Catenin and γ-Catenin. Preparation of genomic DNA and PCR were performed as described (16). For *CTNNB1*, a genomic PCR fragment including codon 1 in exon 2 to codon 90 in exon 4 and encompassing the NH_2-terminal regulatory region was amplified using PCR primers ABS58 and ABS60 (Table 1). For *CTNNG1*, a genomic fragment encoding amino acids 1–57 of γ-catenin and encompassing the NH_2-terminal regulatory region was amplified using PCR primers ABS63 and ABS65. PCR products were gel purified and sequenced directly with internal primers (ABS30 and ABS32 for *CTNNB1* and ABS64 for *CTTNNG1*) using Thermo-Sequenase (Amersham Corp.) and ^{33}P-labeled ddNTPs (Amersham) according to the manufacturer's instructions. Each mutation was verified in both the sense and antisense directions. When possible, the somatic nature of the mutation was established by sequencing genomic DNA derived from normal tissue of the same patient.

Mutational Analysis of APC, GSK-3β, GSK-3α, and Tcf-4. For those cell lines or xenografts with full-length APC expression or without prior evidence of *APC* mutation, codons 680–1693 of *APC* were analyzed for mutations. This region of *APC* was amplified by PCR from genomic DNA in two segments (2 and 3) as described previously (17). Segment 2 was analyzed for mutations by the *in vitro*-synthesized protein assay (17, 18), whereas segment 3 was analyzed for mutations by DNA sequencing (15). In three samples lacking *APC* and β-catenin mutations, the coding regions of *GSK3A*, *GSK3B*, and *TCF-4* were sequenced. *GSK3A* was amplified by RT-PCR in three separate segments using primer pairs AGSK-P1A2/AGSK-P1B, AGSK-P2A2/AGSK-P2B2, and AGSK-P3A/AGSK-P3B. The entire coding region was sequenced using these primers and primers AGSK-S1B, AGSK-S1B3, AGSK-S2B, and AGSK-S3A2. *GSK3B* was amplified by RT-PCR in two separate fragments using primer pairs BGSK-P1A/BGSK-P1B and BGSK-P2A/BGSKP2B. The entire coding region except codons 1–5 was sequenced using these primers and primers BGSK-S1B2 and BGSK-S2B2. *TCF-4* was amplified by RT-PCR in two segments using primers pairs TCF-P1A2/TCF-P1B and TCF-P2A/TCF-P2B. The entire coding region excluding codons 1–16 was sequenced using these primers and primers TCF-S1A1, TCF-S1A2, TCF-S2A1, TCF-S2A2, TCF-S2A3, and TCF-S2B2. During the process of sequencing *TCF-4* segment 2, a previously unidentified alternate splice form was found. The region containing the alternate sequence was amplified, and the two splice variants were isolated by gel electrophoresis and sequenced.

Results and Discussion

β-Catenin Mutations in CR Tumors. Previous analyses of *CTNNB1* identified activating mutations in five CR cancers lacking APC mutations (10) and in a small fraction of unselected CR cancers (19, 20). To define more fully the role of *CTNNB1* mutations in CR tumors, we searched for activating mutations of β-catenin in a larger panel of CR tumors, chosen because it represented the only collection

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[4] The abbreviations used are: APC, adenomatous polyposis coli; CR, colorectal; RT, reverse transcription; MIN, microsatellite instability.

Table 1 *Oligonucleotide primers used for mutational analysis*

Name	Oligonucleotide sequence (5′ to 3′)
ABS58	TACAACTGTTTTGAAAATCCAGCGTGGAC
ABS60	TCGAGTCATTGCATACCTGTCC
ABS30	TTGATGGAGTTGGACATGGC
ABS32	AGTGAAGGAATGAAGAAAATCC
ABS63	CTCAGTAGCCACGATGGAGGTG
ABS65	TTCTTGAGCGTGTACTGGCG
ABS64	ATGGAGCAGCCTATCAAGGTG
AGSK-P1A2	CAGCCTGGGCCTGTGCTC
AGSK-P1B	GTACACTGTCTCGGGCAC
AGSK-P2A2	TATGCGTAAGCTGGACCAC
AGSK-P2B2	GCGTTCGAGATTTGAACACC
AGSK-P3A	ACAATCCGAGAGATGAACCC
AGSK-P3B	CCCAGATGGAAGTGGAAGG
AGSK-S1B	TCTCTTGGGCAGATATTCC
AGSK-S1B3	GTCACCTTCCCGCTGTCAC
AGSK-S2B	TTCGAGATTTGAACACCTTTG
AGSK-S3A2	CCAACTACAGCGAGTTCAAG
BGSK-P1A	GGAAGGAAAAGGTGATTCGC
BGSK-P1B	TCTGGTGCCCTATAGTACC
BGSK-P2A	TGGAAGTGCAAAGCAGCTG
BGSK-P2B	TGACACTCAAGTAACTGGTG
BGSK-S1B2	TAAATCACAGGGAGCGTCTG
BGSK-S2B2	AGAGTGCAGGTGTGTCTCG
TCF-P1A2	CTTCCAAAATTGCTGCTGGTG
TCF-P1B	TCTCTGAAGAGTGCATTCC
TCF-P2A	GAAAGCTCTTAATGCATTCATG
TCF-P2B	ACCAATGAAGTCGATAAACATC
TCF-P1A	TTCTTCCAAAATTGCTGCTGG
TCF-S1A1	AGCTCCTTCTATTCCGAG
TCF-S1A2	AAGTCCCATCGGTCCAGC
TCF-S2A1	TGAACACAGTGAATGTTTCC
TCF-S2A2	GCCAAGTCACAGACTGAGC
TCF-S2A3	TAAGCAGCTGGGAGAGAC
TCF-S2B2	TCAGCAGTTCTCCAGGTC

of tumors in which *APC* had been assessed for mutations by sequencing the entire coding region (15). *CTNNB1* exon 3 was selected for initial analysis because it encodes the NH_2-terminal regulatory domain of β-catenin (codons 29–45) previously found to contain activating mutations (10, 13). Analysis of this region in 40 tumors identified mutations in six cases (Table 2; examples shown in Fig. 1). The somatic nature of each mutation was established by sequencing DNA derived from normal tissue of the same patient (data not shown). Interestingly, *CTNNB1* mutations were detected in two adenomas, including one less than 6 mm in diameter. Furthermore, the incidence of mutations in adenomas (2 of 16) was similar to that in carcinomas (4 of 24). These results indicate that *CTNNB1* mutations, like *APC* mutations, occur early during tumorigenesis and are consistent with an equivalent role for *APC* and *CTNNB1* mutations in CR tumorigenesis.

Further evidence for the functional equivalence of *CTNNB1* and *APC* mutations is provided by the nonoverlapping nature of *APC* and *CTNNB1* mutations. Six of the 12 (50%) CR cancer samples lacking *APC* mutations possessed mutations affecting the NH_2-terminal regulatory domain of β-catenin (Table 2). Conversely, of the 28 tumor samples previously shown to contain APC mutations, none possessed mutations in *CTNNB1* exon 3. This mutually exclusive distribution was unlikely to be due to chance alone ($P < 3 \times 10^{-4}$ by Fisher's exact test). It was interesting that three (C7, C9, and C15) of the six tumors with *CTNNB1* mutations exhibited MIN characteristic of DNA mismatch repair deficiency (data not shown), whereas approximately 15% of unselected CR tumors exhibit MIN (21–23). This is consistent with the suggestion that β-catenin mutations might occur more frequently in MIN tumors (20). However, three (A13, A14, and C17) of the tumors with *CTNNB1* mutations did not exhibit MIN. These results support the notion that although many of the same pathways are affected in MIN tumors, the actual genes targeted may be altered by DNA mismatch repair deficiency.

To further characterize the spectrum of *CTNNB1* mutations in tumors, we analyzed CR cancer cell lines and xenografts that exhibited MIN. A total of 33 MIN-positive lines were evaluated for *CTNNB1* and *APC* mutations. Seven of the fifteen (47%) samples lacking *APC* mutations possessed β-catenin mutations (Table 3). We were able to obtain normal DNA to confirm the somatic nature of four of the seven lesions (data not shown). No mutations of the β-catenin regulatory domain were found among the 18 samples with *APC* mutations (Table 3). Thus, as with the primary CR tumor samples, the panel of cell lines and xenografts exhibited a statistically significant ($P < 2 \times 10^{-3}$ by Fisher's exact test) mutual exclusivity with respect to the distribution of inactivating APC and activating β-catenin mutations, providing further evidence that APC and β-catenin function within the same pathway. Combining the data from the two samples yielded an incidence of β-catenin mutations of 13 of 27 (48%) in tumors with intact APC and 0 of 46 in APC-deficient tumors.

Analysis of *APC*-deficient tumors did not yield mutations affecting residues in β-catenin implicated in regulation by APC and GSK-3β. We did, however, identify a unique somatic mutation (G50D) in *CTNNB1* in one tumor (Cx10) with mutant *APC*. Although this substitution occurs near the regulatory serine/threonine residues, its functional consequences remain unclear at this time. Furthermore, the presence of a glutamate residue at this position in *Drosophila* suggests that an aspartate residue at this position might be minimally disruptive. In addition, Cx10 possesses a relatively late truncating *APC* mutation (frameshift at codon 1935; Ref. 24), which retains an intact

Table 2 *β-Catenin and APC mutations in primary CR tumors*

Sample	APC status[a]	β-Catenin status[b]
Adenomas		
A1	MUT	WT
A2	MUT	WT
A3	MUT/LOH	WT
A4	MUT	WT
A5	LOH	WT
A6	LOH	WT
A7	MUT	WT
A8	WT	WT
A9	MUT	WT
A10	MUT	WT
A11	MUT/LOH	WT
A12	MUT	WT
A13	WT	TCT ⇒ TTT/S45F
A14	WT	ΔExon3
A15	MUT/LOH	WT
A16	WT	WT
Carcinomas		
C1	MUT	WT
C2	MUT	WT
C4	MUT	WT
C5	WT	WT
C6	MUT	WT
C7	WT	TCT ⇒ TTT/S45F
C8	MUT	WT
C9	WT	ACC ⇒ GCC/T41A
C10	MUT	WT
C11	WT	WT
C12	MUT	WT
C13	LOH	WT
C14	MUT	WT
C15	WT	TCT ⇒ TTT/S45F
C16	MUT	WT
C17	WT	GGA ⇒ GTA/G34V
C18	MUT	WT
C19	MUT/LOH	WT
C20	MUT	WT
C21	MUT	WT
C22	MUT	WT
C23	WT	WT
C24	WT	WT
C25	MUT/LOH	WT

[a] The *APC* mutational analyses of these samples has been previously reported. MUT, mutant; indicates the presence of an intragenic somatic *APC* mutation. LOH, loss of heterozygosity at the *APC* locus. WT, wild type; no mutation was detected.

[b] Mutations of the β-catenin regulatory domain were studied.

[c] Previously reported mutation (10).

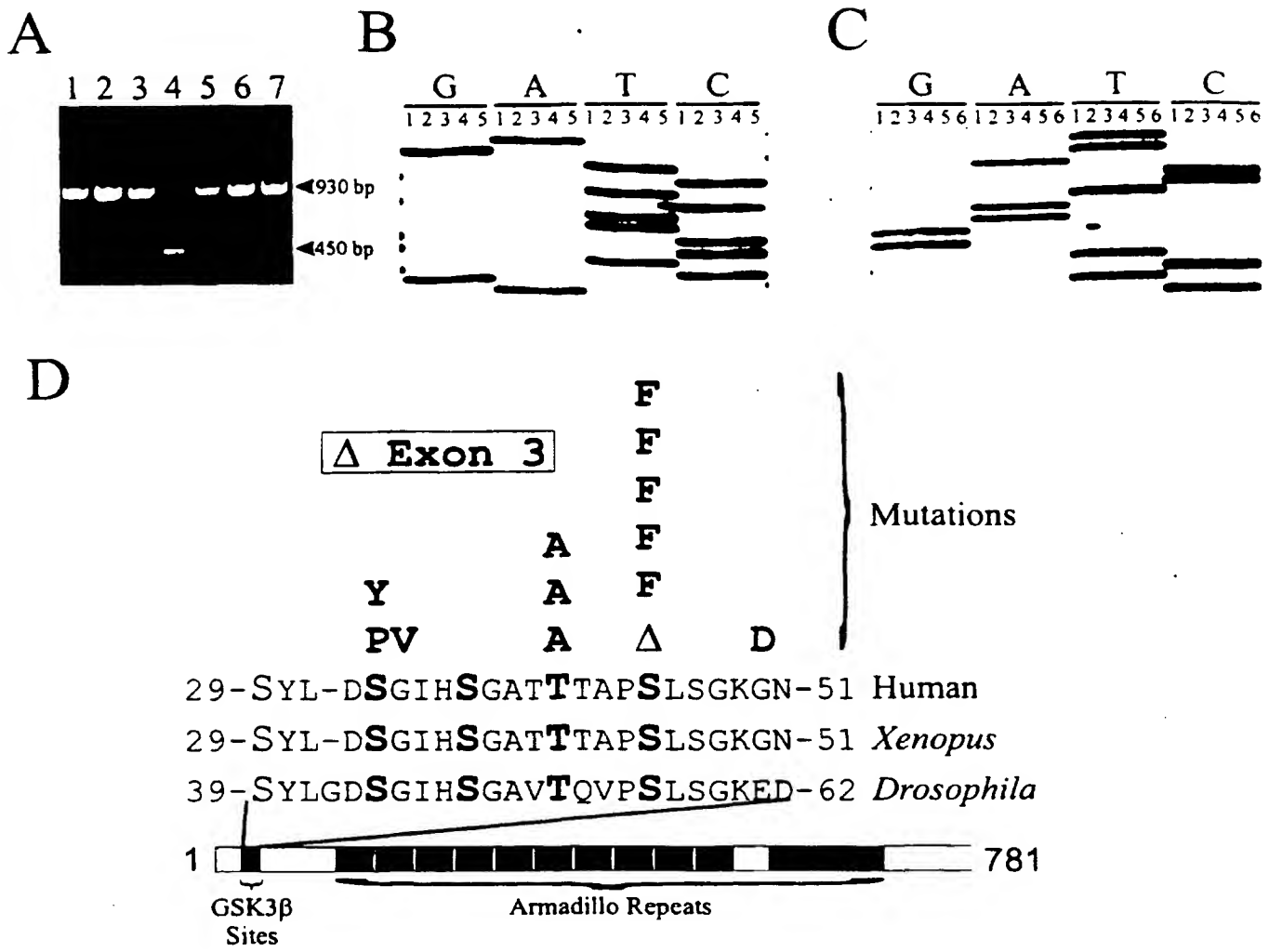

Fig. 1. β-catenin mutations in CR tumors. *A*, PCR of genomic DNA revealing a deletion of exon 3 in adenoma A14. *B*, nucleotide sequencing of the regulatory domain of β-catenin revealed a C→T (S45Y) transition in adenoma A13. *Lanes 1–5* correspond to samples C3, C2, C1, A15, and A13, respectively. Nucleotides 126–139 of β-catenin, as derived from an antisense primer, are shown. The film was flipped to allow the sequence to be read in the sense direction. *C*, nucleotide sequencing of the regulatory domain of β-catenin reveals a G→T (G34V) transversion in Carcinoma C17. *Lanes 1–6* correspond to samples C15, C17, C5, C7, C9, and C23, respectively. Nucleotides 96–109 of β-catenin are shown in the sense direction. *D*, summary of the β-catenin mutations. The regions encompassing the β-catenin regulatory domains from human, frog (*Xenopus*), and fly (*Drosophila*) are shown with the identified mutations indicated above.

series of β-catenin-binding repeats and therefore would be predicted to be only partially defective at down-regulating β-catenin levels.

With the lone exception noted above, each of the 14 *CTNNB1* mutations listed in Tables 2 and 3 is readily rationalized with respect to its effect upon β-catenin stability and, consequently, upon signaling. Eleven of the lesions are point mutations or small deletions affecting one of a series of five serines or threonines found near the NH_2 terminus of β-catenin (Fig. 1*D*). These residues have been implicated as substrates for GSK-3β and are thought to play a central role in the down-regulation of β-catenin levels by GSK-3β (25). Thus, loss of these phosphorylation sites likely blocks down-regulation by GSK-3β and APC. The remaining two *CTNNB1* mutations also likely affect β-catenin regulation by APC. One of the mutations is an intragenic deletion spanning part of intron 2, all of exon 3, and part of intron 3 (Fig. 1*A*). This deletion is predicted to encode a β-catenin protein lacking amino acids 5–80, encompassing the entire NH_2-terminal regulatory region; similar deletions have been shown previously to stabilize β-catenin (12). The other mutation results in a G→V substitution at amino acid 34 (Fig. 1*C*). This residue occurs within the NH_2-terminal regulatory region of β-catenin in a motif (DSGΨXS, where Ψ indicates the hydrophobic residue) that is shared with IκBα, is conserved across species (fly to human), and has recently been shown to be critical for ubiquinin-dependent proteosomal degradation of both β-catenin and IκBα (26, 27, 32). Thus, mutation of G34 likely yields a protein that is refractory to proteosomal degradation, thereby rendering it insensitive to APC regulation.

Recently, *CTNNB1* mutations within armadillo repeats 2 and 3 and outside the NH_2-terminal regulatory domain were described in two CR cell lines with mutant *APC* (19). However, the effects of this type of mutation on β-catenin are not known, and no mutation outside the regulatory domain has been shown to activate β-catenin/Tcf transcription.

γ-Catenin Mutations in CR Tumors. The above analyses indicate that *CTNNB1* mutations occur in almost half of CR tumors lacking APC mutations. If activation of the APC/β-catenin/Tcf signaling pathway is critical for tumor initiation, mutations in other components of the pathway might be involved in the genesis of the remaining CR cancers. One candidate for such a component is γ-catenin, which shares strong amino acid similarity with β-catenin. In particular, the NH_2-terminal regulatory motif is conserved between

the two proteins. In addition, both proteins bind APC, α-catenin, E-cadherin, and Tcf (5, 28, 29). Finally, γ-catenin exhibits signaling activity similar to that of β-catenin in *Xenopus* (30). Thus, activating mutations in γ-catenin could reasonably be expected to substitute for loss of APC. To address this possibility, we sequenced the NH_2-terminal regulatory region (codons 17–50) of γ-catenin. Analysis of 33 tumors, including 13 of the 14 tumors lacking both *APC* and *CTNNB1* mutations, revealed no mutations in the NH_2-terminal regulatory region of γ-catenin (data not shown).

Detailed Analysis f the APC/β-Catenin/Tcf Pathway. The above analyses indicate that γ-catenin regulatory domain mutations do not occur in tumors lacking APC or β-catenin mutations. To explore other potential mechanisms of activating β-catenin signaling, we performed a more detailed mutational analysis in selected cancer cell lines (RG, KS, and Vaco 486) lacking APC and β-catenin regulatory domain mutations. Considering the possibility that mutations outside the regulatory domain might activate β-catenin, we sequenced the entire coding region of *CTNNB1* in each of the lines. No mutations were detected. Because GSK-3β, like APC, is predicted to inhibit β-catenin signaling, we considered the possibility that GSK-3β inactivation could substitute for APC or β-catenin mutation. We therefore sequenced the coding region of GSK-3β and its homologue, GSK-3α, in the same three cell lines with intact APC and β-catenin, but we did not identify any somatic mutations in these genes. Thus, either other components of the pathway are altered in these lines or activation of β-catenin signaling is not absolutely required for CR tumorigenesis.

If the ultimate effect of APC and β-catenin mutations is activation of Tcf-mediated transcription, then there should be an absolute requirement for Tcf in CR tumors. Previously, we found that 43 of 43 CR cell lines expressed the Tcf family member Tcf-4 (9). As expected, sequencing of *TCF-4* in three lines with intact β-catenin and APC revealed no mutations. However, this analysis did reveal an alternative spliced form of *TCF-4* representing 50% of the transcripts expressed in CR cells (Fig. 2).

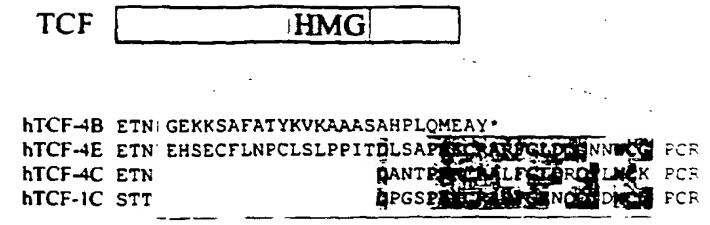

Fig. 2. Splice forms of Tcf-4. The similarity between the newly described Tcf-4C and Tcf-1C splice form is shown. Also shown are the Tcf-4B and Tcf-4E splice forms. The predominant Tcf-4 splice forms in human CR cancers appear to be Tcf-4C and Tcf-4E.

The above results suggest that *CTNNB1* mutations can uniquely substitute for APC mutations and that they account for approximately half of CR cancers with intact APC. The remaining cancers cannot easily be explained by mutations of γ-catenin, GSK-3α, or GSK-3β. Although other components of the pathway may be altered in these tumors, there may be instances in which constitutive activation of β-catenin/Tcf signaling is not required, suggesting either that downstream components are mutated or that parallel pathways are targeted. Answers to these questions should become apparent when the downstream targets of β-catenin/Tcf signaling are better defined. Further analysis of CR cancers lacking mutations in known components of the APC/β-catenin/Tcf pathway may prove valuable in this regard.

Table 3 *β-Catenin and APC mutations in CR carcinoma cell lines*

Line	APC status[a]	β-Catenin status[b]
SW48	FL[c]	TCT ⇒ TAT/S33Y[d]
LS180	WT	TCT ⇒ TTT/S45F
RKO	FL[c]	WT
DLD1	MUT	WT
VACO6	FL[c]	WT
HCT116	FL[c]	ΔTCT/Δ45S[d]
VACO143	MUT	WT
MIP106	MUT	WT
LIM1215	WT	ACC ⇒ GCC/T41A
LOVO	MUT	WT
HCT115	MUT	WT
VACO5	MUT	WT
C	MUT	WT
VACO432	MUT	WT
VACO444	MUT	WT
VACO457	MUT	WT
VACO481	MUT	WT
CO95	WT	TCT ⇒ CCT/S33P
KM12	WT	WT
CX2	WT	WT
CX7	MUT	WT
CX10	MUT	GGC ⇒ GAC/G50D
CX12	WT	WT
MX14	MUT	WT
MX17	WT	WT
MX23	WT	ACC ⇒ GCC/T41A
HX9	WT	WT
HX16	MUT	WT
HX37	MUT	WT
HX39	MUT	WT
HX41	WT	WT
HX43	WT	TCT ⇒ TTT/S45F
HX45	MUT	WT

[a] FL, full-length; indicates the presence of a full-length APC protein. MUT, mutant; indicates the presence of a truncating *APC* mutation. WT, wild type; no *APC* mutation was detected and protein status is unknown.

[b] Mutations of the β-catenin regulatory domain were studied. WT indicates that no mutation was detected in this region.

[c] Previously reported analysis (10, 24, 31).

[d] Previously reported mutation (10).

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